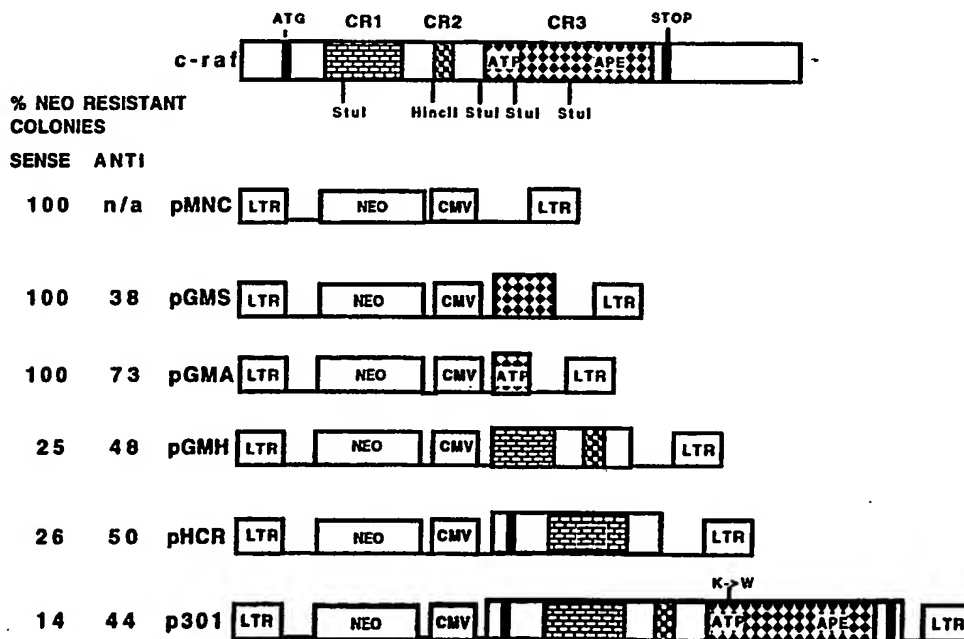




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(21) International Application Number: PCT/US92/07002 (22) International Filing Date: 21 August 1992 (21.08.92) (30) Priority data: 07/748,931 23 August 1991 (23.08.91) US (71) Applicant: THE UNITED STATES OF AMERICA represented by THE SECRETARY, DEPARTMENT OF HEALTH AND HUMAN SERVICES [US/US]; Washington, DC (US). (72) Inventors: RAPP, Ulf, R. ; 5226 39th Street, N.W., Washington, DC 20015 (US). APP, Harald ; 908-3B Gatepost Lane, Frederick, MD 21702 (US). STORM, Stephen, M. ; 1418B Taney Avenue, #302, Frederick, MD 21702 (US).		(74) Agents: NEEDLE, William, H. et al.; Needle & Rosenberg, 133 Carnegie Way, N.W., Suite 400, Atlanta, GA 30303 (US). (81) Designated States: AU, CA, JP, European patent (AT, BE, CH, DE, DK, ES, FR, GB, GR, IE, IT, LU, MC, NL, SE). Published <i>With international search report.</i>

(54) Title: RAF PROTEIN KINASE THERAPEUTICS**(57) Abstract**

It is a general object of this invention to provide a DNA segment comprising a Raf gene in an antisense orientation downstream of a promoter. It is a specific object of this invention to provide a method of inhibiting Raf expression comprising expressing an antisense Raf gene in a cell such that said Raf expression is inhibited. It is a further object of the invention to provide a method of inhibiting Raf kinase activity comprising replacement of a serine or threonine amino acid within the Raf gene for a non-phosphorylated amino acid.

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RAF PROTEIN KINASE THERAPEUTICS

BACKGROUND OF THE INVENTION

Field of the Invention

5 The present invention relates, in general, to methods of inducing a therapeutic effect. In particular, the present invention relates to therapeutic uses of Raf protein kinases.

Background Information

10 Raf serine- and threonine-specific protein kinases are cytosolic enzymes that stimulate cell growth in a variety of cell systems (Rapp, U.R., et al. (1988) In The oncogene handbook; T. Curran, E.P. Reddy, and A. Skalka (ed.) Elsevier Science Publishers; The Netherlands, pp.213-253; Rapp, U.R., et al. (1988) Cold Spring Harbor Sym. Quant. Biol. 53:173-184; Rapp, U.R., et al. (1990) In: Curr. Top. Microbiol. Immunol. Potter and Melchers (eds), Berlin, Springer-Verlag 166:129-139). Three
15 isozymes have been characterized: c-Raf (Raf-1) (Bonner, T.I., et al. (1986) Nucleic Acids Res. 14:1009-1015). A-Raf (Beck, T.W., et al. (1987) Nucleic Acids Res. 15:595-609), and B-Raf (Ikawa, S., et al. (1988) Mol. Cell. Biol. 8:2651-2654; Sithanandam, G. et al. (1990) Oncogene 5:1775). These enzymes differ in their expression in
20 various tissues. Raf-1 is expressed in all organs and in all cell lines that have been examined, and A- and B-Raf are expressed in urogenital and brain tissues, respectively (Storm, S.M. (1990) Oncogene 5:345-351).

 Raf genes are proto-oncogenes: they can initiate
30 malignant transformation of cells when expressed in specifically altered forms. Genetic changes that lead to oncogenic activation generate a constitutively active protein kinase by removal or interference with an N-terminal negative regulatory domain of the protein
35 (Heidecker, G., et al. (1990) Mol. Cell. Biol. 10:2503-

2512; Rapp, U.R., et al. (1987) In Oncogenes and cancer S. A. Aaronson, J. Bishop, T. Sugimura, M. Terada, K. Toyoshima, and P.K. Vogt (ed.) Japan Scientific Press, Tokyo). Microinjection into NIH 3T3 cells of
5 oncogenically activated but not wild-type versions of the Raf-protein prepared with Escherichia coli expression vectors results in morphological transformation and stimulates DNA synthesis (Rapp, U.R., et al. (1987) In Oncogenes and cancer; S. A. Aaronson, J. Bishop, T.
10 Sugimura, M. Terada, K. Toyoshima, and P.K. Vogt (ed.) Japan Scientific Press, Tokyo; Smith, M.R., et al. (1990) Mol. Cell. Biol. 10:3828-3833). Thus, activated Raf-1 is an intracellular activator of cell growth. Raf-1 protein serine kinase is a candidate downstream effector of
15 mitogen signal transduction, since Raf oncogenes overcome growth arrest resulting from a block of cellular ras activity due either to a cellular mutation (ras revertant cells) or microinjection of anti-ras antibodies (Rapp, U.R., et al. (1988) In The Oncogene Handbook, T. Curran,
20 E.P. Reddy, and A. Skalka (ed.), Elsevier Science Publishers; The Netherlands, pp.213-253; Smith, M.R., et al. (1986) Nature (London) 320:540-543).

c-Ras function is required for transformation by a variety of membrane-bound oncogenes and for growth
25 stimulation by mitogens contained in serum (Smith, M.R., et al. (1986) Nature (London) 320:540-543). Raf-1 protein serine kinase activity is regulated by mitogens via phosphorylation (Morrison, D.K., et al. (1989) Cell 58:648-657), which also effects subcellular distribution
30 (Olah, Z., et al. (1991) Exp. Brain Res. 84:403; Rapp, U.R., et al. (1988) Cold Spring Harbor Sym. Quant. Biol. 53:173-184).

Raf-1 activating growth factors include platelet-derived growth factor (PDGF) (Morrison, D.K., et
35 al. (1988) Proc. Natl. Acad. Sci. USA 85:8855-8859), colony-stimulating factor 1 (Baccarini, M., et al. (1990) EMBO J. 9:3649-3657), insulin (Blackshear, P.J., et al.

(1990) J. Biol. Chem. 265:12131-12134; Kovacina, K.S., et al. (1990) J. Biol. Chem. 265:12115-12118), epidermal growth factor (EGF) (Morrison, D.K., et al. (1988) Proc. Natl. Acad. Sci. USA 85:8855-8859), interleukin 2 (Turner, B.C. et al. (1991) Proc. Natl. Acad. Sci. USA 88:1227), and interleukin 3 and granulocyte-macrophage colony-stimulating factor (Carroll, M.P., et al (1990) J. Biol. Chem. 265:19812-19817). Upon mitogen treatment of cells, the transiently activated Raf-1 protein serine kinase translocates to the perinuclear area and the nucleus (Olah, Z., et al. (1991) Exp. Brain Res. 84:403; Rapp, U.R., et al. (1988) Cold Spring Harbor Sym. Quant. Biol. 53:173-184). Cells containing activated Raf are altered in their pattern of gene expression (Heidecker, G., et al. (1989) In Genes and signal transduction in multistage carcinogenesis, N. Colburn (ed.), Marcel Dekker, Inc., New York. pp. 339-374), and Raf oncogenes activate transcription from Ap-1/PEA3-dependent promoters in transient transfection assays (Jamal, S., et al. (1990) Science 344:463-466; Kaibuchi, K., et al. (1989) J. Biol. Chem. 264:20855-20858; Wasylyk, C., et al. (1989) Mol. Cell. Biol. 9:2247-2250).

There are at least two independent pathways for Raf-1 activation by extracellular mitogens: one involving protein kinase C (KC) and a second initiated by protein tyrosine kinases (Blackshear, P.J., et al. (1990) J. Biol. Chem. 265:12131-12134; Kovacina, K.S., et al. (1990) J. Biol. Chem. 265:12115-12118; Morrison, D.K., et al. (1988) Proc. Natl. Acad. Sci. USA 85:8855-8859; Siegel, J.N., et al. (1990) J. Biol. Chem. 265:18472-18480; Turner, B.C. et al. (1991) Proc. Natl. Acad. Sci. USA 88:1227). In either case, activation involves Raf-1 protein phosphorylation. Raf-1 phosphorylation may be a consequence of a kinase cascade amplified by autophosphorylation or may be caused entirely by autophosphorylation initiated by binding of a putative activating ligand to the Raf-1 regulatory domain,

analogous to PKC activation by diacylglycerol (Nishizuka, Y. (1986) Science 233:305-312).

SUMMARY OF THE INVENTION

It is a general object of this invention to
5 provide a construct comprising a DNA segment comprising a Raf gene in an antisense orientation downstream of a promoter.

It is a specific object of this invention to
provide a method of inhibiting Raf expression comprising
10 expressing an antisense Raf gene in a cell such that said Raf expression is inhibited.

It is a further object of the invention to
provide a method of inhibiting Raf kinase activity
comprising replacing a serine or threonine amino acid
15 within the Raf gene for an amino acid not susceptible to phosphorylation.

Further objects and advantages of the present invention will be clear from the description that follows.

BRIEF DESCRIPTION OF THE DRAWINGS

20 Figure 1. Schematic diagram of murine and human c-raf-1 cDNAs and expression plasmids used. GMA and GMS contain Stu1 restriction fragments of the mouse c-raf-1 cDNA. HCR an N-terminal HincII fragment of the human cDNA. p301 consists of all the coding sequence of a
25 mutant human c-raf-1 cDNA. The lysine(375) to tryptophan (K → W) mutation in the ATP-binding site is indicated (Heidecker, G. et al. Molec. Cell. Biol. 10:2503-2512 (1990)). Restriction fragments were cloned in both sense and antisense orientation. NIH/3T3 cells were transfected
30 with sense and antisense plasmids, and with the pMNC vector as control. G418-resistant (400 µg ml⁻¹) colonies containing more than 50 cells were counted after three

weeks. The pMNC vector served as internal standard. The experiment was repeated three times (twice for HCR) with different batches of plasmid preparations. Variations between experiments were in the order of 10% but did not affect the ratios between the different constructs shown. CR1-3, conserved regions; ATP, ATP-binding domain; LTR, mouse Moloney virus long terminal repeat; NEO, neomycin-resistance gene; CMV, cytomegalovirus immediate early promoter.

Figure 2. Morphological reversion of raf-transformed cells by transfection with raf antisense and mutant constructs. a. p48 raf-transformed 208/F12 (Schultz, A.M. et al. Oncogene 2:187-193 (1988)) or b. v-raf-transformed F4 (Rapp, U.R., et al. Proc. Natl. Acad. Sci. U.S.A. 80:4218-4222 (1983)) fibroblasts were transfected with plasmids p301-1 (sense) and 301-2 (antisense) or GMS-7 (sense) and GMS-8 (antisense), respectively, as well as with the pMNC vector. Monolayer growth with minor irregularities and a decreased ability to form soft agar colonies was categorized as partial reversion. Flat clones showed no areas of overgrowth and did not form colonies in soft agar. c. A representative analysis of Raf protein expression in individual cell clones. t. Transformed; im. intermediate; f. flat (clone GMS-8/2); f* (clone GMS-8/3).








Figure 3. Mitogen responsiveness and proliferative capacity of Raf-depleted cells. a. DNA synthesis induced by serum or TPA in serum-starved cells is depicted as the number of nuclei incorporating ³H-thymidine. b. Long term growth curves GMS-7 is a pool of 10 clones transfected with sense DNA. GMS-8/2 and GMS-8/3 are flat clones reverted with antisense DNA. a, , starved cells; , TPA-induced cells; , SERUM-induced cells. b, , F4; , GMS-7b; , GMS-8/2; , GM-8/3.

Figure 4. Time course of Raf-1 mobility shift upon growth factor treatment. Cells (10^7) expressing either wild-type (HER14) or kinase-negative (DK721A) EGF-R were stimulated at 37°C with 40 nM EGF for the times indicated, lysed, and subjected to immunoprecipitation with anti-SP63 polyclonal antiserum. Immunoprecipitated proteins were separated by 7.5% SDS-PAGE, transferred to nitro-cellulose, and probed with the same antiserum. Immunoreactive proteins were detected with ^{125}I -labeled protein A, and autoradiographs were exposed for 12 h. Each lane represents immunoprecipitates from 10^7 cells. Lanes: 2 through 5. HER14 cell; 7 and 8, DK721A cells: 9 and 10, DK721A cells with competing SP63 peptide (10 $\mu\text{g/ml}$); 1, 6, 9, and 11, marker proteins of 97 and 67 kDa.

Figure 5. Kinase activity upon EGF treatment HER14 and DK721A cells. Monolayer cultures of HER14 or K721A cells were incubated in the presence or absence of 40 nM EGF for 10 min at 37°C. Lysates were centrifuged, and the resulting supernatants were immunoprecipitated with Raf-1 antiserum. Immunocomplexes were assayed for kinase activity using peptide (IVQQFGFQRRASDDGKLTG) as substrate. In the absence of peptide, immune complex kinase assays with unstimulated cells yielded $\leq 5\%$ of counts observed in the peptide assay with stimulated cells. No counts were incorporated when a modified version of this peptide was used, in which serine in position 12 was replaced by alanine, and position 5 retained the Raf-1-specific tyrosine.

Figure 6. Association of Raf-1 with ligand-activated EGF-R in HER14, DK721A, or A431 cells. Density-arrested and serum-starved HER14, DK721A, or A431 cells were stimulated for 10 min with 40 nM EGF at 37°C before lysis with RIPA buffer and immunoprecipitation. (A) Immunoprecipitates from HER14 and DK721A cells with anti-v-Raf 30K polyclonal antiserum or with a monoclonal EGF-R

antibody (108) were subjected to 7.5% SDS-PAGE and transferred to nitrocellulose. EGF-R was detected by incubating with a polyclonal antiserum (RK2) against the EGF receptor (Margolis, B., et al. (1989) Cell 57:1101-1107), followed by ¹²⁵I-labeled protein A labeling. Exposure times for immunoblots were 3 days (lanes 1 through 4) or 1 day (lanes 5 and 6). (B and C) Immunoprecipitates from EGF-treated and control A431 cells with Raf-1 specific anti-v-Raf 30K antiserum or monoclonal anti-EGF-R antibody 108 were blotted, and the blots were developed sequentially with EGF-R antiserum RK2 (B) and anti-v-Raf 30K (C). Exposure times were 3 days and 1 day for panels B and C, respectively.

Figure 7. Phosphoamino acid analysis of the immunoprecipitated Raf-1 protein from EGF-treated and untreated HER14 cells, HER14 cells (10⁷) were phosphate starved for 16h, labeled with 1 mCi of [³²P]phosphate for 3 h at 37°C, and treated with 40 nM EGF for 10 min at 37°C. Cells were lysed in RIPA buffer and immunoprecipitated. Proteins were separated by 7.5% SDS-PAGE, the Raf-1 bands were cut out of the gel, and the protein was electroeluted. From the electroeluted Raf-1 protein 1,960 cpm was recovered from the EGF-treated cells and 1,111 cpm was recovered from the untreated cells. The proteins were hydrolyzed for 2 h at 110°C in 6 N hydrochloric acid. Phosphoamino acid analysis was performed at pH 1.8 as described by Coover et al. (Cooper, A.A., et al. (1983) Methods in Enzymol. 99:387-402). The Raf-1 protein showed a shift in mobility when part of the electroeluted protein was rerun on 7.5% SDS-PAGE.

Figure 8. Independence of EGF-mediated Raf-1 activation from PKC. HER14 cells (10⁷) were incubated for 48 h with or without 200 ng TPA and stimulated with either 100 ng of TPA for 20 min at 37°C. Cells were lysed in RIPA buffer, equal amounts of protein were

immunoprecipitated with anti-v-Raf 30-kDa antiserum and electrophoresed, and the separated proteins were blotted onto nitrocellulose. The blot was incubated with the same antibody and then labeled with ¹²⁵I-labeled protein A.

5

DETAILED DESCRIPTION OF THE INVENTION

The present invention relates to the Raf protein kinase inhibitors and methods of use thereof.

In one embodiment, the present invention relates to antisense expression constructs comprising a Raf protein
10 kinase gene. Raf-1 function is inhibited by expressing c-raf-1 antisense RNA or kinase-defective c-raf-1 mutants. Antisense RNA for c-raf-1 interferes with proliferation of normal NIH/3T3 cells and reverts raf-transformed cells. In revertant cells, DNA replication induced by serum or
15 TPA is eliminated or reduced proportionately to the reduction in Raf protein levels. Expression of a kinase-defective Raf-1 mutant (craf301) or a regulatory domain fragment (HCR) inhibits serum-induced NIH/3T3-cell proliferation and raf transformation even more
20 efficiently. Inhibition by antisense RNA or craf301 blocks proliferation and transformation by Ki- and Ha-ras oncogenes. Thus, raf functions as an essential signal transducer downstream of serum growth factor receptors, protein kinase C and ras.

25 In another embodiment, the present invention relates to a method of inhibiting Raf expression comprising expressing an antisense Raf gene (more specifically, Raf-1) in a cell such that said Raf expression is inhibited.

In another embodiment, the present invention relates
30 to inhibitory peptides of Raf derived from Raf kinase specific substrate sequences. Phosphorylation sites of Raf substrates can be determined which are expected to yield consensus phosphorylation site motifs for the various Raf isozymes. Studies which gave rise to the
35 present invention demonstrate that Raf is the subject of

autophosphorylation. In one preferred embodiment, consensus substrate peptides are altered by introduction of alanine for phosphorylation targets serine or threonine.

5 In another preferred embodiment, the present invention relates to a method of inhibiting Raf kinase activity comprising replacing a codon within the Raf gene encoding a serine or threonine amino acid for a codon encoding an amino acid not susceptible to phosphorylation
10 and transforming said gene into a cell such that said Raf activity is inhibited.

In yet another embodiment, the present invention relates to a method of inhibiting Raf kinase activity comprising modifying Raf by replacing a serine or
15 threonine amino acid within Raf for an amino acid not susceptible to phosphorylation and delivering said modified Raf to a cell such that said Raf expression is inhibited.

The present invention is described in further detail in the following non-limiting examples (see Kolch
20 W. et al (1991) Nature 349:426 and App et al. (1991) Molec. Cell. Biol. 11(2):913-919).

EXAMPLES

The following protocols and experimental details are referenced in the Examples that follow:

25 Expression plasmid construction. pMNC digested with XhoI and BamHI was blunt ended with T4 DNA polymerase. The mouse and human cDNAs were cut with StuI or HincII, respectively, and appropriate sized fragments were ligated with the pMNC vector. GMA contains residues 1254-1426 and
30 GMS 1427-1697 of the mouse cDNA, HCR 1-903 of the human c-raf-1 cDNA (Bonner et al. Nucleic Acids Res. 14:1009-1015 (1986)). The translation termination codon for HCR sense is provided by vector sequences resulting in the addition of nine amino acids. To construct p301-1 (sense

orientation), an EcoRI-XbaI fragment of p628 (Bonner et al. Nucleic Acids Res. 14:1009-1015 (1986)) encompassing the coding sequence of a human c-raf-1 cDNA was cloned into BluescriptKS (Stratagene). Lysine(375) was changed
5 to tryptophan by site-directed mutagenesis resulting in the creation of a unique BamHI site. This cDNA was transferred into the SacI-XhoI sites of pSVL (Pharmacia), then cloned into the XhoI-BamHI sites of pMNC as an XhoI-BamHI (partial digest) fragment. The corresponding
10 antisense plasmid, p301-2, was generated by cloning the blunt-ended c-raf 301 EcoRI-XbaI fragment into blunt-ended pMNC.

Western analysis with PBB1. Cells were lysed in TBST (160 mM NaCl, 20 mM Tris HCl, pH7.5, 2 mM EDTA, 1% Triton X-
15 100, 1 mM PMSF). Lysates were adjusted to equal protein concentrations (Biorad protein assay kit). Raf proteins were precipitated with the monoclonal antibody PBB1 and analyzed by western blotting with the polyclonal serum #137 as described previously (Kolch, W. et al. Oncogene
20 6:713-720 (1990)).

Mitogen responsiveness and proliferative capacity assay. 10^4 cells were plated on cover slips and serum-starved for 24 h before incubation with 20% fetal calf serum (Gibco) or 100 ng ml⁻¹ TPA (Sigma). 14 h after addition of
25 mitogens, cells were labelled with 1 μ Ci ml⁻¹ ³H-thymidine for 5 h. Cells were counter-stained with Giemsa and labelled nuclei were counted. For long-term growth curves. 10^5 cells were seeded in six-well plates in DMEM medium supplemented with 10% FCS. Each day, one well was
30 trypsinized and counted with a Coulter cell counter. All determinations were performed in triplicate.

Cell maintenance. NIH 3T3 clone 2.2 cells devoid of endogenous EGF-R were transfected with wild-type (HER14) or kinase-negative (DK721A) receptors as described

previously (Honegger, A.M., et al. (1987) Cell 51:199-209; Honegger, A.M. (1987) Mol. Cell. Biol. 7:4567-4571; Margolis, B., et al. (1989) Cell 57:1101-1107). In the case of the kinase-negative receptor mutant, the putative ATP binding lysine was substituted by an alanine (Honegger, A.M., et al. (1987) Cell 51:199-209; Honegger, A.M. (1987) Mol. Cell. Biol. 7:4567-4571). Cells were maintained in Dulbecco modified Eagle medium (DMEM) containing 10% (vol/vol) calf serum.

Preparations of cytosolic cell extracts and immunoprecipitations. Cells were grown in 75-cm² flasks in DMEM containing 10% calf serum until confluency and starved overnight in 0.05% calf serum. Before lysis, cells were exposed to 40 nM EGF for 0 min at 37°C and rinsed three times in phosphate-buffered saline. Control cells were not exposed to EGF. Cells were lysed in TBST buffer (50 mM Tris hydrochloride (pH 7.3), 150 mM NaCl, 0.5% Triton X-100) or in RIPA buffer (50 mM Tris hydrochloride (pH 7.3), 150 mM NaCl, 1% Triton X-100, 0.5% desoxycholate, 0.1% sodium dodecyl sulfate (SDS), 5 mM EDTA, 1 mM dithiothreitol, 0.2 mM sodium orthovanadate, 25 mM sodium fluoride, 10 mM sodium pyrophosphate, 25 mM glycerophosphate). Insoluble material was removed by centrifugation at 4°C for 30 min at 12,000 x g. Protein concentrations were determined by the method of Bradford (Bradford, M.M. (1976) Anal. Biochem. 72:248-254). Immunoprecipitations were performed by incubating lysates with polyclonal rabbit antiserum against the v-Raf 30-kDa protein (Kolch, W., et al. (1988) Biochim. Biophys. Acta 949:233-239) or a polyclonal rabbit antiserum against a synthetic peptide (SP63) corresponding to the last 12 carboxy-terminal amino acids of the Raf-1 protein and protein A for 3h at 4°C.

Western immunoblotting. The immunoprecipitates were resolved by SDS-polyacrylamide gel electrophoresis (PAGE).

The gels were electroblotted on nitrocellulose, and the blots were blocked with 5% (wt/vol) gelatin in TBST buffer and incubated with polyclonal antiserum against Raf-1 or EGF-R. After extensive washing with TBST buffer, the blot was labeled with ^{125}J -staph protein A (Dupont NEN). Nonbound ^{125}J -staph was removed by washing the blots with TBST buffer, and the dried membrane was exposed to x-ray film.

Immunocomplex kinase assay. Immunoprecipitates were washed three times with cold RIPA buffer and twice with kinase buffer (50 mM Tris hydrochloride (pH 7.3), 150 mM NaCl, 12.5 mM MnCl_2 , 1 mM dithiothreitol, and 0.2% Tween 20). Immunocomplex kinase assays were performed by incubating immunoprecipitates from 10^6 cells in 80 μl of kinase buffer with 20 μCi of $[\gamma\text{-}^{32}\text{P}]\text{ATP}$ (10 mCi/ml) and 20 μl of the Raf-1 substrate peptide (5 mg/ml) for 30 min at 25°C . The sequence of the Raf-1 substrate peptide is IVQQFGFQRRASDDGKLTD. A control peptide had tyrosine in position 5, as does wild-type Raf-1, and alanine in place of serine in position 12. The assay was linear for at least 40 min. The phosphorylation reaction was terminated by spotting 15- μl aliquots of the assay mixture on a 2- by 2-cm Whatman P81 phosphocellulose filter. The filters were washed four times for 30 min in 1% orthophosphoric acid and air dried, and the amount of ^{32}P incorporated was determined by the Cerenkov method. No differences were observed when counts were compared between filters on which the whole reaction mix or only the supernatant was spotted. Peptide phosphorylation in this assay was verified by running the reaction products on 20% SDS gels.

Phosphoamino acid analysis. One-dimensional phosphoamino acid analysis was carried out as described by Cooper et al. (Cooper, A.A., et al. (1983) Methods in Enzymol. 99:387-402). Phosphoamino acids were separated at pH 1.8 (6% formic acid and 15% acetic acid) for 4 h at 750 V.

EXAMPLE 1Antisense Experiments

Portions of c-raf-1 cDNAs were expressed in sense and antisense orientation using the pMNC vector (Figure 1). After transfection into NIH/3T3 cells the number of neomycin-resistant colonies was scored. Antisense constructs yielded roughly half the number of colonies as did the corresponding sense construct or the vector control, indicating that raf antisense RNA interferes with viability and/or proliferation. As NIH/3T3 cells express no B-raf and 10-fold less A-raf than Raf-1 (Storm, S.M. et al. Oncogene 5:345-351 (1990)), the effect can be ascribed to interference with Raf-1. Antisense colonies were generally smaller and grew slower than sense or vector control colonies. Ten out of ten antisense colonies showed barely detectable levels of Raf-1 protein, whereas levels in sense control clones were unchanged. An alternative approach to Raf-1 inhibition used inactive mutants (Rapp, U.R. et al. The Oncogene Handbook (eds. E.P. Reddy, A.M. Skalka and T. Curran) 213-253 (Elsevier Science. The Netherlands, 1988); Heidecker, G. et al. Molec. Cell. Biol. 10:2503-2512 (1990)). A truncated Raf-1 protein (HCR) corresponding to conserved region 1 reduced colony numbers fourfold. A kinase-defective Raf-1 mutant protein, craf301 (plasmid p301-1), was even more efficient, decreasing colony yield about sevenfold (Heidecker, G. et al. Molec. Cell. Biol. 10:2503-2512 (1990)). The surviving colonies from these experiments could not be maintained as stable cell lines. raf-transformed cell-lines were examined for morphological reversion and inhibition of proliferation. p301 constructs were transfected into 208-F12 fibroblasts which overexpress a transforming mouse Raf-1 protein (Schultz, A.M. et al. Oncogene 2:187-193 (1988)). p301-2 caused partial or complete reversion of the transformed phenotype in approximately half the transfectants. Reversion

correlated with loss of anchorage-independent growth. Again, p301-1 was more efficient than p301-2 (Figure 2a). These clones were unstable, but cell-lines sufficiently stable for biochemical analysis were obtained after pGMS
5 transfection of v-raf transformed cells, F4 (Rapp, U.R., et al. Proc. Natl. Acad. Sci. U.S.A. 80:4218-4222 (1983)). Neither pMNC nor the control plasmid GMS-7 was effective, whereas the antisense construct, GMS-8, completely or partially reverted F4 (Figure 2b). Reduction of raf mRNA
10 and protein levels correlated with the extent of reversion (Figure 2c). In one clone, GMS-8/3 (marked f* in Figure 2c), raf protein expression was undetectable. These cells grew extremely slowly, arresting at 50-60% confluency, and eventually died.

15 To measure the effects of raf-protein depletion on the mitogen response, the ability of serum and TPA to induce DNA synthesis in serum-starved cells was determined (Figure 3a). F4 and GMS-7 cells synthesize DNA independently of mitogens. Constitutive DNA synthesis was
20 diminished in GMS-8/2, which retained an inducible response similar to NIH/3T3 cells. GMS-8/3 was completely blocked in constitutive and TPA-inducible DNA replication. Serum-stimulation of GMS-8/3 was reduced seven-fold, and long-term growth was also severely diminished (Figure 3b).

25 v-Ki-ras-transformed NIH/3T3 cells were transfected with the p301 plasmids (Table 1a). p301-1 and p301-2 reduced neomycin (neo)-resistant colony yield to a similar degree as in NIH/3T3 cells (Figure 1), suggesting that Raf-1 is required for proliferation of ras-
30 transformed cells. Morphological reversion of established ras-transformed cells was less efficient than of raf-transformed cells (Figure 2). To test the effect of raf-inhibition on the initiation of ras-transformation, a constant amount of v-Ha-ras (pSV2neo/ras, Clanton, D.J. et
35 al. Molec. Cell. Biol. 7:3092-3097 (1987)) plasmid was co-transfected with an equal or four-molar amount of the p301 vectors (Table 1b). Although the neomycin resistance of

pmNC-based plasmids accounted for a background of flat
neo[®] colonies that presumably did not express pSV2neo/ras,
transfection with p301 vectors markedly increased the
number of morphological revertants at the expense of
5 transformed colonies. The inhibition was dose-dependent
and almost complete at four-molar excess of p301-1.

Thus, NIH/3T3 cells Raf-1 kinase functions
downstream of membrane receptors and ras proteins and is
essential for growth-induction by serum factors and
10 protein kinase C. Membrane receptor systems can now be
examined individually for Raf-1-dependence by inhibition
with the blocking constructs described herein.
Furthermore, the proposed position of raf in the
communication pathway between cell membrane and nucleus
15 makes raf an attractive target for the design of novel
antiproliferative agents, especially as this data show
that raf inhibition is dominant over transformation by ras
and by implication by other non-nuclear oncogenes.

TABLE I.
raf-inhibition blocks ras-mediated proliferation
 and transformation

5	a) <u>v-Ki-ras</u> cell transfection				
	Yield of <u>neo</u> ^r Morphology of <u>neo</u> ^r colonies				
	Plasmids	colonies	flat	intermediate	transformed
	pMNC ¹	100 ± 0%	0 ± 0%	0 ± 1%	100 ± 1%
10	pMNC301-2 ²	61 ± 8%	2 ± 1%	15 ± 7%	83 ± 7%
	pMNC301-1 ³	30 ± 7%	15 ± 3%	15 ± 5%	70 ± 6%
	b) NIH/3T3 co-transfection with <u>v-Ha-ras</u> (pSV2neo/ <u>ras</u>) and p301				
	Plasmids (ratio 1:1)	inhibition ⁴	flat	Morphology of <u>neo</u> ^r colonies intermediate	transformed
15	<u>ras</u> + pMNC ¹	0 ± 3%	27 ± 3%	17 ± 5%	56 ± 3%
	<u>ras</u> + p301-2 ²	53 ± 4%	28 ± 1%	46 ± 9%	26 ± 7%
	<u>ras</u> + p301-1 ³	61 ± 3%	46 ± 4%	32 ± 7%	22 ± 4%
	Plasmids (ratio 1:4)				
20	<u>ras</u> + pMNC ¹	0 ± 1%	33 ± 4%	23 ± 3%	44 ± 3%
	<u>ras</u> + p301-2 ²	61 ± 5%	48 ± 5%	35 ± 7%	17 ± 4%
	<u>ras</u> + p301-1 ³	84 ± 4%	67 ± 4%	25 ± 6%	7 ± 3%

Cells were transfected and G418-resistant (400 µg ml⁻¹) colonies were morphologically examined according to the criteria described in Fig. 2. Percentages are calculated for two experiments with ≥ 200 (a) or ≥ 400 (b) colonies per transfection.

¹ vector control

² antisense orientation

³ sense orientation

⁴ The efficiency of inhibition or ras transformation is given as percentage reduction in the number of

transformed colonies.

EXAMPLE 2

Association and Kinase Activity of Raf-1 with the EGF Receptor

To determine whether EGF induces the shift in migration in SDS gels that is typical for phosphorylation activation of Raf-1 protein kinase, lysates of treated and control cells were subjected to immunoprecipitation and immunoblotting with Raf-1-specific antiserum. NIH 3T3 cells lacking endogenous EGF-R but expressing approximately 3×10^5 human wild-type (HER14) EGF-R or kinase-negative mutant K721A EGF-R were transferred to starvation medium (0.05% calf serum) at early confluency and stimulated with EGF at 40 nM for 0 to 10 min. The effect of EGF on Raf-1 mobility is shown in Figure 4. In the absence of EGF treatment, Raf-1 migrates as a single polypeptide of 72 kDa, corresponding to the expected molecular mass of Raf-1 protein kinase (Bonner, T.I., et al. (1986) Nucleic Acids Res. 14:1009-1015). The addition of EGF to HER14 but not to K721A cells resulted in a small increase in apparent mass of Raf-1 to 74 kDa. This shift first became detectable by 5 min, when approximately 50% of Raf-1 protein was affected, and continued to spread so that by 10 min the entire pool of Raf-1 protein had been modified. The inability of EGF to induce the Raf mobility shift in NIH 3T3 cells expressing the kinase-negative mutant of EGF-R demonstrates that receptor dimerization is not sufficient for Raf-1 modification, since the point mutation in K721A does not affect this event (Ulrich, A., et al. (1990) Cell 61:203-212). It therefore seemed likely that the kinase activity of the EGF-R was important in mediating induction of the mobility shift in Raf-1.

The increase in apparent molecular mass of Raf-1 protein upon EGF treatment was due to phosphorylation, since incubation with potato acid phosphatase completely reversed the gel retardation. To evaluate the effect of EGF-stimulated raf-1 protein phosphorylation on its serine- and threonine-specific protein kinase activity, immune complex kinase assays were performed that utilized a synthetic peptide (IVQQFGFQRRASDDGKLTD) or histone H1 as a substrate. The peptide corresponds to a potential autophosphorylation site in the Raf-1 kinase, which has been altered by substitution of phenylalanine for tyrosine in position 7 so as to restrict it from tyrosine phosphorylation.

For kinase assays, lysates of HER14 and K721A cells were prepared before and after stimulation with 40 nM EGF for 10 min. Comparison of the levels of kinase activity in Raf-containing immunoprecipitates showed a sixfold stimulation in HER14 cells upon EGF treatment (Figure 5). Similar data were obtained when histone H1 was used as a substrate. Consistent with the absence of the EGF-induced mobility shift of Raf-1 in NIH 3T3 cells expressing the kinase-negative mutant for of the EGF-R, no stimulation of Raf-1 protein kinase activity was observed in K721A cells (Figure 5). When Raf-1 kinase activity was assayed with a modified version of the substrate peptide in which Ser-12 was replaced by alanine and Tyr-5 was retained, no counts were detected on the spotted filters. This indicates that the kinase activity measured by the assay did not include a contribution of a contaminating tyrosine kinase activity.

Activity EGF-R associates with the candidate signal transducing enzyme PLC γ (Margolis, B., et

al. (1990) Mol. Cell. Biol. 10:435-441; Margolis, B., et al. (1989) Cell 57:1101-1107; Meisenhelder, J., et al. (1989) Cell 57:1109-1112; Wahl, M., et al. (1989) Proc. Natl. Acad. Sci. USA 86:1568-1572). Similarly, Raf-1 was shown to coimmunoprecipitate with activated PDGF- β receptor in cell lines expressing high levels of receptors (Morrison, D.K., et al. (1989) Cell 58:648-657). To evaluate whether ligand-induced activation of Raf-1 protein kinase by the EGF-R correlated with receptor association, two cell systems were used: the NIH 3T3 cells expressing wild-type and mutant receptors (Figure 6A) and human A431 cells (Figure 6B) expressing approximately 2×10^6 EGF-R per cell (15, 16). Serum-starved cells were stimulated with 40 nM EGF for 10 min. and lysates from cells were immunoprecipitated with Raf-1 or EGF-R specific antibodies. After separation by SDS-PAGE and transfer to nitrocellulose, immunoblotting was performed with either anti-EGF-R or anti-Raf-1 antibodies. EGF-R is present in anti-Raf-1 antibody immunoprecipitates from EGF-treated cells (Figure 6). The coprecipitating EGF-R in HER14 cells has a decreased mobility on PAGE, compared with that of the EGF-R from untreated controls (Figure 6A, lanes 4 and 6); this decreased mobility was previously demonstrated to be due to ligand-induced autophosphorylation (Margolis, B., et al. (1989) Cell 57:1101-1107). Cells expressing the kinase-negative mutant receptor KD712A did not show the mobility shift in the EGF-R upon EGF treatment and lacked EGF stimulation of EGF-R Raf-1 coimmunoprecipitation. A small amount of unshifted EGF-R was detected in Raf-1 immunoprecipitates from all cells; this EGF-R could be reduced by preclearing with preimmune serum. EGF-R can be coprecipitated in lysates

from EGF-treated A431 cells, whereas there is not EGF-R present in immunoprecipitates from untreated cells (Figure 6). Sequential reprobing of the Western blot with polyclonal Raf-1 rabbit antiserum (Figure 6B) indicates that a small fraction (~1%) of the EGF-R associates with shifted Raf-1. Furthermore, the blot demonstrates that the EGF-R-Raf-1 association was not due to unequal loading of the gel with Raf-1 immunoprecipitates. Estimates from three independent experiments indicate that the fraction of immunoprecipitable EGF-R protein that is present in Raf-1 antibody precipitates from EGF-treated HER14 or A431 cells is on the order of 1.0%.

Considering the observed association of Raf-1 protein with activated EGF-R as well as the EGF-induced mobility shift of Raf-1, it might be expected that the receptor-associated fraction of Raf-1 was phosphorylated on tyrosine. The immunoblots from experiments in Figure 6 were therefore reprobed with antiphosphotyrosine antibodies. The antibodies readily detected EGF-induced tyrosine phosphorylation of the EGF-R, PLC γ , GAP, and other unknown substrates (Ulrich, A., et al. (1990) Cell 61:203-212), but no tyrosine phosphorylated bands in the size range of Raf-1 protein were detected. The experiment was scaled up to examine the presence of tyrosine-phosphorylated Raf-1 protein in anti-Raf or anti-EGF-R antibody immunoprecipitates from 10^8 HER14 cells per lane; again, tyrosine phosphorylation of Raf-1 could not be detected. Consistent with the absence of anti-phosphotyrosine antibody-reactive Raf-1 protein, phosphoamino acid analysis of Raf-1 from EGF-treated cells did not reveal any phosphotyrosine (Figure 7). For this experiment,

10⁷ HER14 cells were labeled with ³²Pi, and the Raf-1 proteins were immunoprecipitated with anti-v-Raf 30-kDa polyclonal antiserum and subjected to SDS-PAGE. Phospholabeled Raf-1 protein was excised from the gel, electroeluted, and hydrolyzed in 6 N HCl. The only labeled phosphoamino acid detectable was phosphoserine; thus it can be concluded that EGF induced an increase in serine phosphorylation of c-Raf (Figure 7). When the same experiment was done with A431 cells, trace amounts of phosphotyrosine were detected that were independent of EGF treatment. The lower limit for detection of phosphotyrosine in Raf-1 in these experiments was on the order of 1% of phosphoserine.

The absence of tyrosine phosphorylation of Raf-1 protein in response to EGF in HER14 cells raises the possibility that serine protein kinase(s) acts as an intermediate in a kinase cascade connecting the stimulated EGF-R to activation of Raf-1 kinase. One candidate for this role is PKC, since this enzyme has previously been shown, upon treatment of cells with tetradecanoylphorbol-13-acetate (TPA), to trigger Raf-1 phosphorylation and kinase activation (Morrison, D.K., et al. (1988) Proc. Natl. Acad. Sci. USA 85:8855-8859; Siegel, J.N., et al. (1990) J. Biol. Chem. 265:18472-18480). It was therefore examined whether EGF induction of the Raf-1 mobility shift was dependent on the presence of PKC (Figure 8). HER14 cells were pretreated with 200 ng of TPA for 72 h for complete downregulation of PKC and then tested for their ability to respond to EGF with Raf-1 retardation. The PKC down-regulation by pretreatment with TPA was effective in eliminating the TPA-induced Raf-1 retardation. In contrast, EGF-induced Raf-1

mobility shift was not blocked by down-regulation of PKC.

* * * * *

5 All publications mentioned hereinabove
are hereby incorporated in their entirety by
reference. In particular, Kolch W et al (1991)
Nature 349:426-428 and App H et al (1991)
Molecular and Cellular Biology 11(2):913-919 are
hereby incorporated in their entirety by
10 reference.

While the foregoing invention has been
described in some detail for purposes of clarity
and understanding, it will be appreciated by one
skilled in the art from a reading of this
15 disclosure that various changes in form and detail
can be made without departing from the true scope
of the invention and appended claims.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i) APPLICANT: Rapp, Ulf R.
App, Harald
Storm, Stephen M.

(ii) TITLE OF INVENTION: RAF PROTEIN KINASE THERAPEUTICS

(iii) NUMBER OF SEQUENCES: 1

(iv) CORRESPONDENCE ADDRESS:

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(E) COUNTRY: USA
(F) ZIP: 20036

(v) COMPUTER READABLE FORM:

(A) MEDIUM TYPE: Floppy disk
(B) COMPUTER: IBM PC compatible
(C) OPERATING SYSTEM: PC-DOS/MS-DOS
(D) SOFTWARE: PatentIn Release #1.0, Version #1.25

(vi) CURRENT APPLICATION DATA:

(A) APPLICATION NUMBER:
(B) FILING DATE:
(C) CLASSIFICATION:

(viii) ATTORNEY/AGENT INFORMATION:

(A) NAME: Scott, Watson T.
(B) REGISTRATION NUMBER: 26,581
(C) REFERENCE/DOCKET NUMBER: WTS/5683/82731/SRL

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(B) TELEFAX: (202) 822-0944
(C) TELEX: 6714627 CUSH

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:

(A) LENGTH: 19 amino acids
(B) TYPE: amino acid
(D) TOPOLOGY: linear

(xi) SEQUENCE DESCRIPTION: SEQ ID NO:1:

Ile Val Gln Gln Phe Gly Phe Gln Arg Arg Ala Ser Asp Asp Gly Lys
1 5 10 15

Leu Thr Asp

WHAT IS CLAIMED IS:

1. A construct comprising a DNA segment comprising a Raf gene in an antisense orientation downstream of a promoter.

2. The construct according to claim 1 wherein said Raf gene is Raf-1.

3. A method of inhibiting Raf expression comprising expressing an antisense Raf gene in a cell under conditions such that said Raf expression is inhibited.

4. The method according to claim 3, wherein said Raf gene is Raf-1.

5. A method of inhibiting Raf kinase activity comprising:

replacing a codon within the Raf gene encoding a serine or threonine amino acid for a codon encoding an amino acid not susceptible to phosphorylation and

transforming said gene into a cell such that said Raf activity is inhibited.

6. The method according to claim 5, wherein said amino acid not susceptible to phosphorylation is alanine.

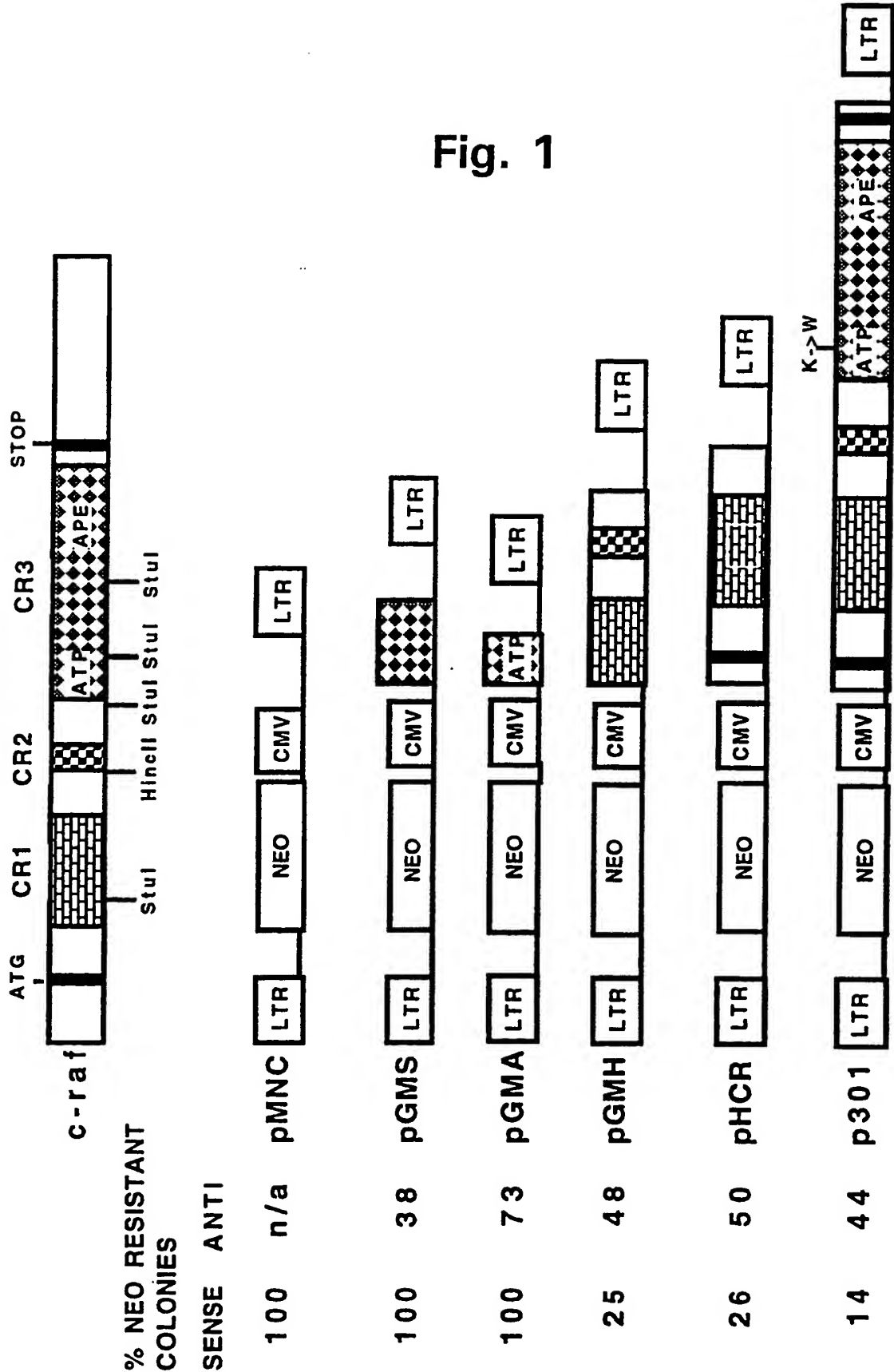
7. A method of inhibiting Raf kinase activity comprising:

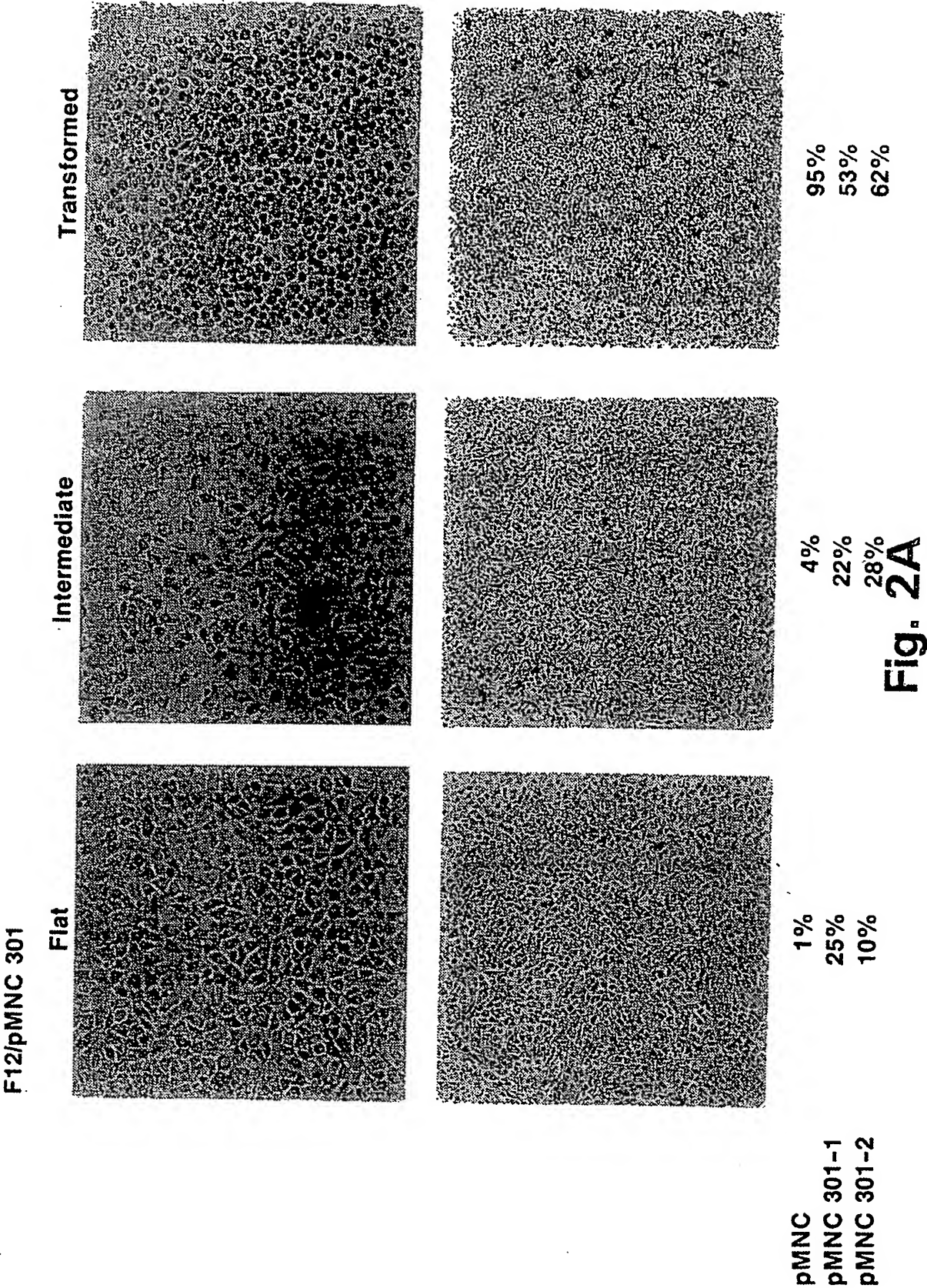
modifying Raf by replacing a serine or threonine amino acid within Raf for an amino acid not susceptible to phosphorylation and

delivering said modified Raf to a cell such that said Raf expression is inhibited.

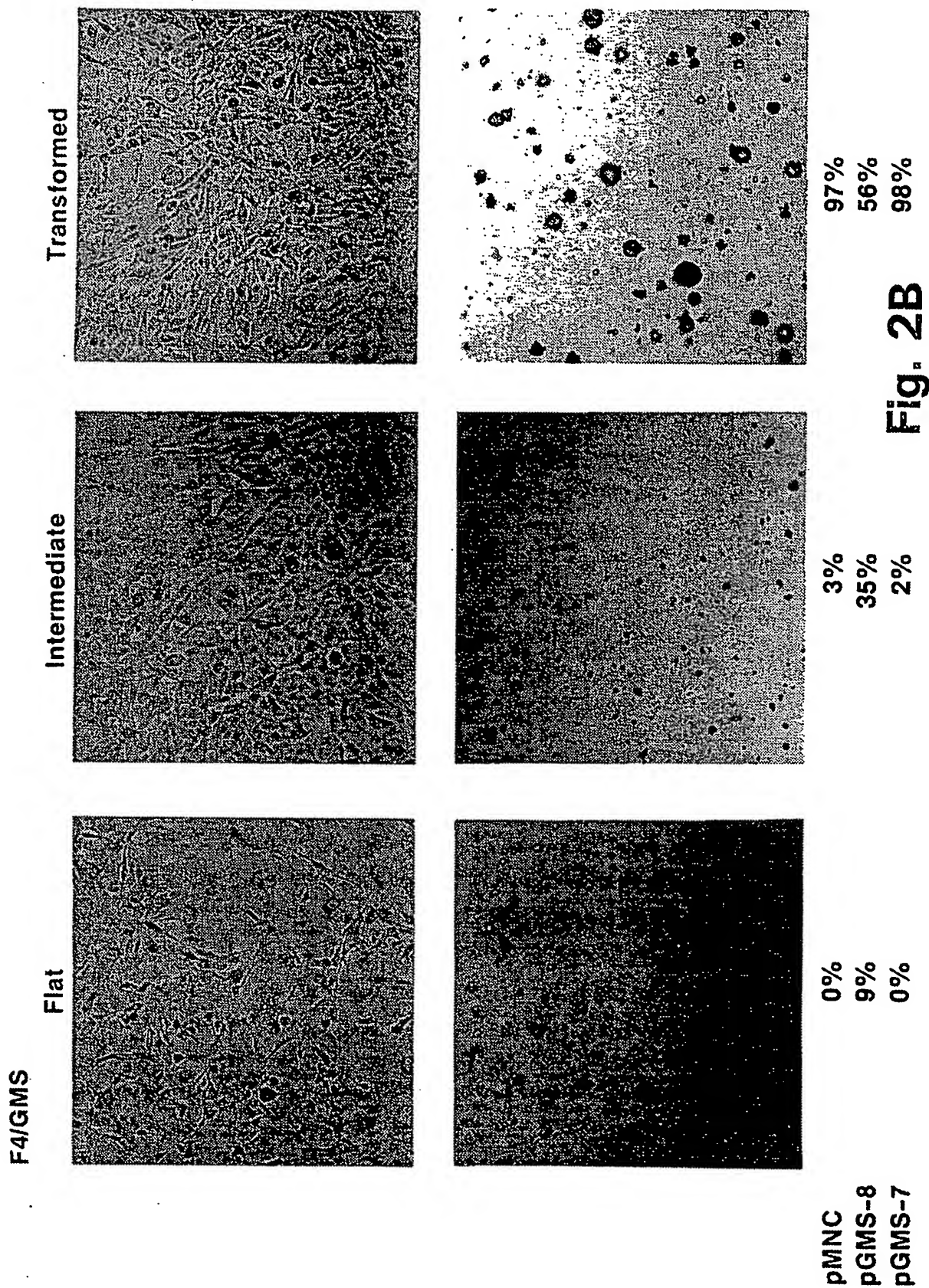
8. The method according to claim 7, wherein said amino acid not susceptible to phosphorylation is alanine.

Fig. 1





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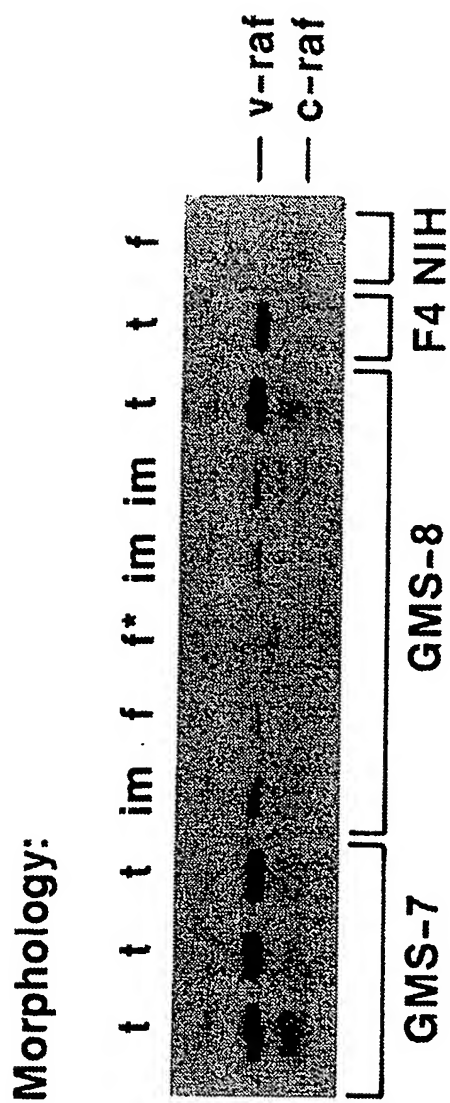
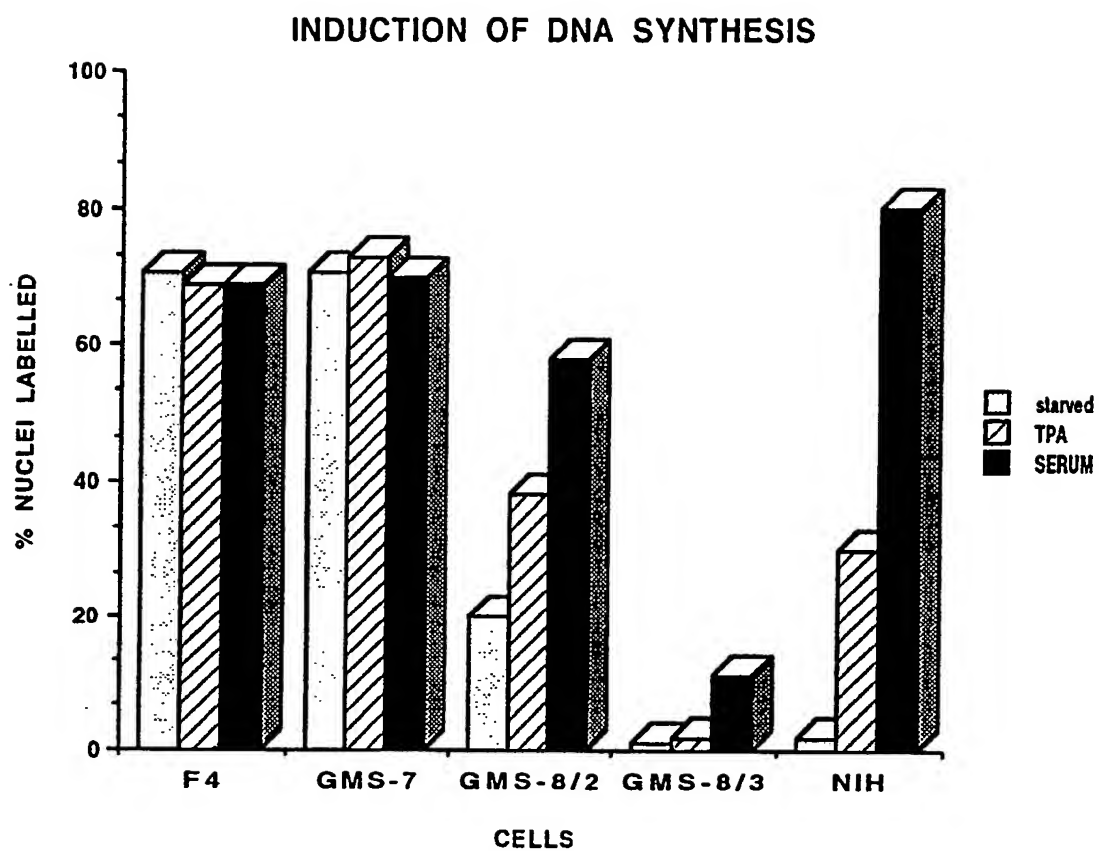
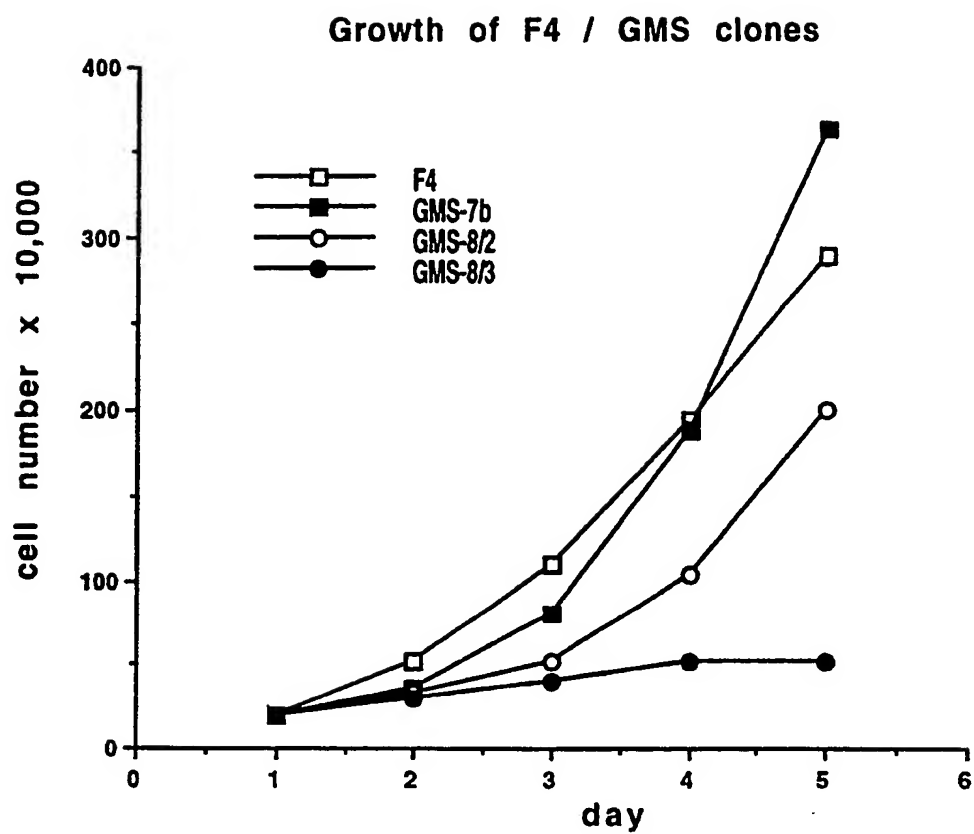


Fig. 2C

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**Fig. 3A****SUBSTITUTE SHEET**

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**Fig. 3B**

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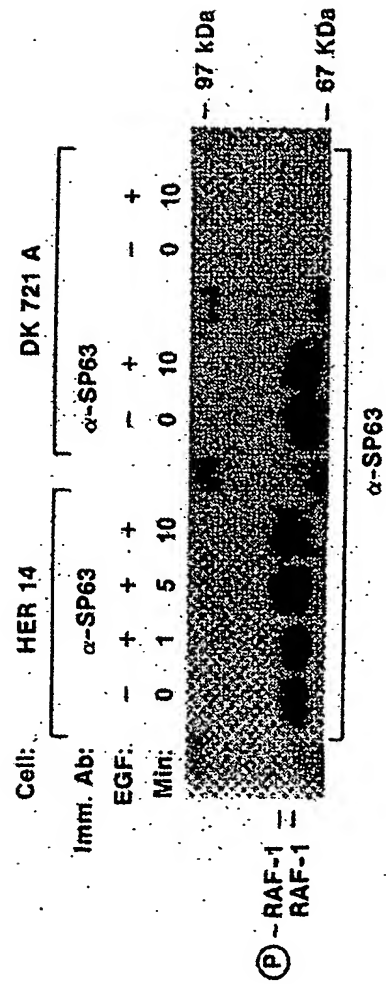
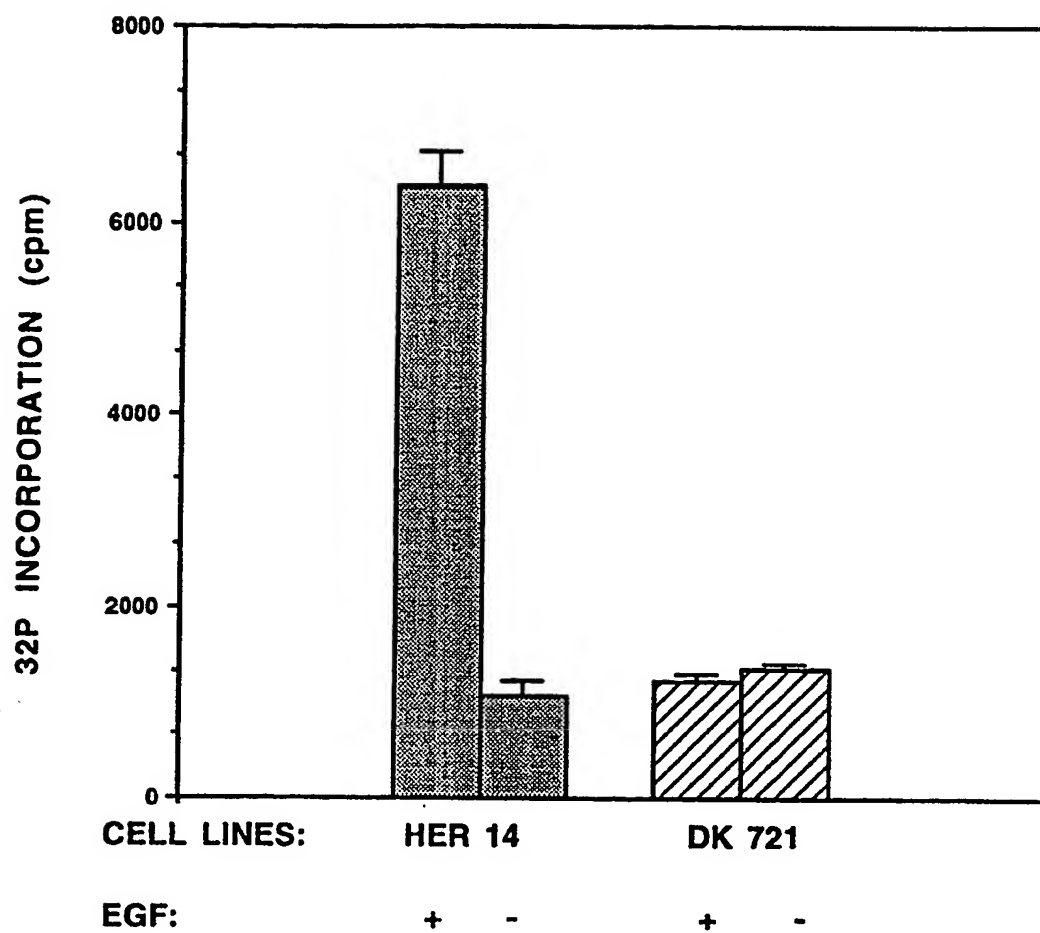


Fig. 4

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**Fig. 5**

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Fig. 6A

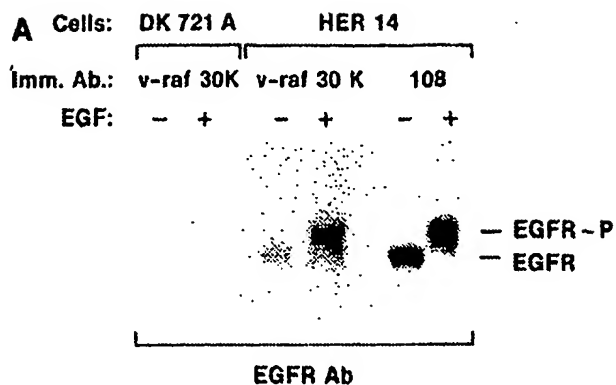


Fig. 6B

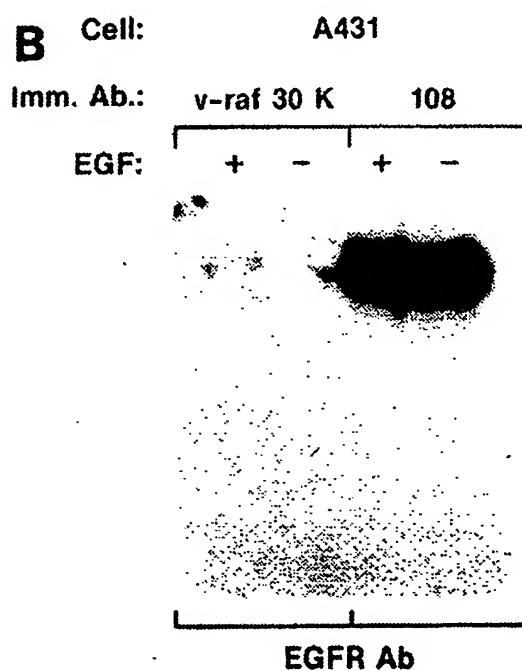
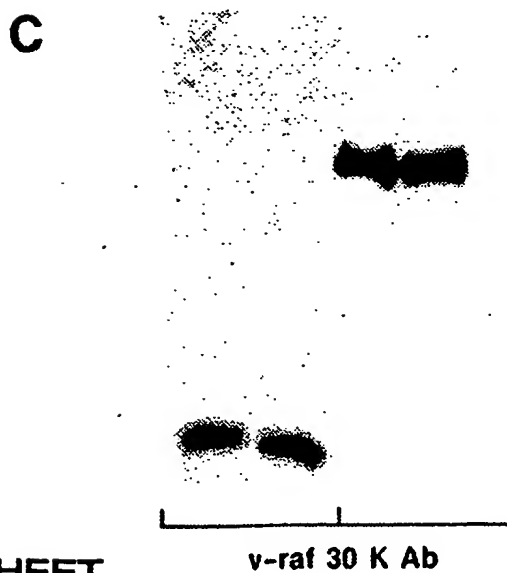


Fig. 6C



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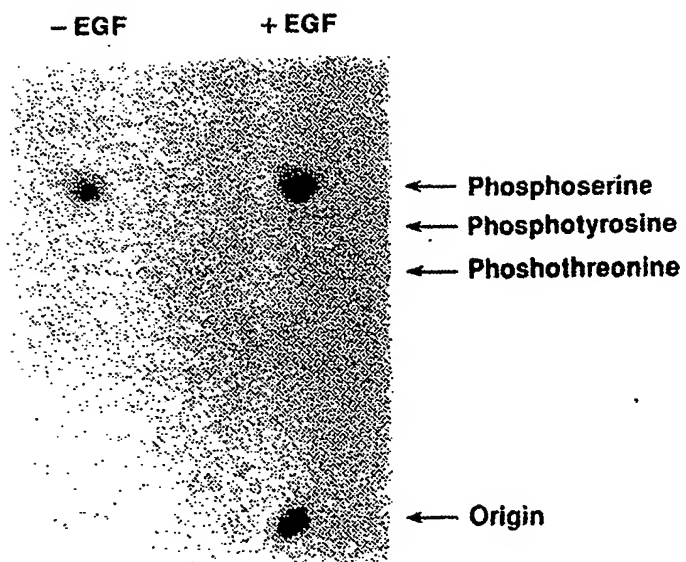


Fig. 7

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100 ng TPA (10 min)	+	-	+	-
200 ng TPA (72 hr)	-	+	+	+
40 nM EGF (10 min)	-	-	-	-

**Fig. 8**

INTERNATIONAL SEARCH REPORT

International Application No

PCT/US 92/07002

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) ⁶		
According to International Patent Classification (IPC) or to both National Classification and IPC		
Int.Cl. 5 C12N15/11; C12N15/12; A61K31/70; A61K37/02		
II. FIELDS SEARCHED		
Minimum Documentation Searched ⁷		
Classification System	Classification Symbols	
Int.Cl. 5	C12N ; C07K ; A61K	
Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched ⁸		
III. DOCUMENTS CONSIDERED TO BE RELEVANT⁹		
Category ¹⁰	Citation of Document, ¹¹ with indication, where appropriate, of the relevant passages ¹²	Relevant to Claim No. ¹³
X	NATURE vol. 349, 31 January 1991, LONDON GB pages 426 - 428 KOLCH, W. ET AL. 'Raf-1 protein kinase is required for growth of induced NIH/3T3 cells' cited in the application see the whole document ---	1-8
X	MOLECULAR AND CELLULAR BIOLOGY vol. 11, no. 2, February 1991, WASHINGTON US pages 913 - 919 APP, H. ET AL. 'Epidermal growth factor (EGF) stimulates association and kinase activity of Raf-1 with EGF receptor' cited in the application see page 915, line 3 - line 9 ---	5-8
<div style="display: flex; justify-content: space-between;"> <div> <p>¹⁰ Special categories of cited documents:¹⁰</p> <p>"A" document defining the general state of the art which is not considered to be of particular relevance</p> <p>"E" earlier document but published on or after the international filing date</p> <p>"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)</p> <p>"O" document referring to an oral disclosure, use, exhibition or other means</p> <p>"P" document published prior to the international filing date but later than the priority date claimed</p> </div> <div> <p>"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention</p> <p>"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step</p> <p>"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.</p> <p>"&" document member of the same patent family</p> </div> </div>		
IV. CERTIFICATION		
Date of the Actual Completion of the International Search		Date of Mailing of this International Search Report
30 NOVEMBER 1992		14, 12, 92
International Searching Authority		Signature of Authorized Officer
EUROPEAN PATENT OFFICE		ANDRES S.M.

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)		
Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No.
Y	<p>PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF USA vol. 88, no. 4, 15 February 1991, WASHINGTON US pages 1227 - 1231 TURNER, B. ET AL. 'Interleukin 2 induces tyrosine phosphorylation and activation of p72-74 Raf-1 kinase in a T-cell line' cited in the application see in particular page 1228, left column: 'IL-2 stimulation results in elevated Raf-1 kinase activity.'</p> <p>---</p>	5-8
Y	<p>JOURNAL OF BIOLOGICAL CHEMISTRY vol. 265, no. 21, 25 July 1990, BALTIMORE, MD US pages 12115 - 12118 KOVACINA, K.S. ET AL. 'Insulin activates the kinase activity of the Raf-1 proto-oncogene by increasing its serine phosphorylation' cited in the application see the whole document</p> <p>---</p>	5-8
A	<p>JOURNAL OF BIOLOGICAL CHEMISTRY vol. 265, no. 21, 25 July 1990, BALTIMORE, MD US pages 12131 - 12134 BLACKSHEAR, P.J. ET AL. 'Insulin activates the Raf-1 protein kinase' cited in the application see the whole document</p> <p>-----</p>	5-8

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 92/07002

Box I Observations where certain claims were found unsearchable (Continuation of item 1 of first sheet)

This international search report has not been established in respect of certain claims under Article 17(2)(a) for the following reasons:

1. ☒ Claims Nos.:
because they relate to subject matter not required to be searched by this Authority, namely:
Remark: Although claims 3-8 (as far as in vivo methods are concerned) are directed to a method of treatment of the human/animal body the search has been carried out and based on the alleged effects of the compound/composition.
2. ☐ Claims Nos.:
because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out, specifically:
3. ☐ Claims Nos.:
because they are dependent claims and are not drafted in accordance with the second and third sentences of Rule 6.4(a).

Box II Observations where unity of invention is lacking (Continuation of item 2 of first sheet)

This International Searching Authority found multiple inventions in this international application, as follows:

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims.
2. ☐ As all searchable claims could be searched without effort justifying an additional fee, this Authority did not invite payment of any additional fee.
3. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims for which fees were paid, specifically claims Nos.:
4. ☐ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claims Nos.:

Remark on Protest

- ☐ The additional search fees were accompanied by the applicant's protest.
- ☐ No protest accompanied the payment of additional search fees.

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